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Synergistic action of vitamin C and amino acids on vitamin E in inhibition of the lipoperoxidation of linoleic acid in disperse systems

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Abstract

The synergistic effect of some amino acids (L-tryptophan, L-cysteine, L-alanine and glycine), glutathione, *n*-butylamine and of vitamin C on the antioxidant effect of vitamin E on the lipoperoxidation of linoleic acid in sodium dodecylsulphate micellar solutions and oil in water emulsions was examined at pH 5.0 and 7.0. The antioxidant activity of vitamin E, measured by oxygen consumption, was similar in both emulsions and micellar solutions. The addition of vitamin C produced only a slight synergistic effect at pH 5.0 and none, or a prooxidative effect, at pH 7.0. The amino acids studied exerted a synergistic effect only at pH 5.0, and their effectiveness was partially related to their lipophilicity. © 1997 Elsevier Science B.V.

Keywords: Linoleic acid; Amino acids; Lipoperoxidation; Vitamin C; Vitamin E; Synergistic effect; Micellar solutions; Emulsions

1. Introduction

The oxidation of polyunsaturated fatty acids in lipids and its inhibition have always received much attention, in connection with the pathological effects of such oxidation, which include inflammation (Kuehl and Egan, 1980), platelet aggregation, asthma and anaphylaxis (Salomon et al., 1978). Peroxidation of membrane lipids has been indicated as one of the primary events in oxidative cellular damage (Plaa and Witschi, 1976).

Antioxidative substances occurring in biological material, and synergistic relationships between them, have long been studied, with a view to protecting against fat oxidation in vivo, for example in food (Marcuse, 1962). Cosmetics and phar-

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maceuticals generally contain primary antioxidants, such as tocopherols, able to break oxidative chain reactions, but a number of substances shown to act synergistically to prevent rancidification are also of interest. several substances which participate as synergists in preventing rancidification are also important.

Amino acids act in varying ways as antioxidants of fats, (Siechowski, 1970; Farag et al., 1978): by rendering innocuous substances prooxidative, or by regenerating oxidized primary antioxidants (Cillard and Cillard, 1986). Systems such as oil-in-water emulsions can influence the behaviour of lipoperoxidation reactions (Riisom et al., 1980; Allan et al., 1979). Several patented antioxidant mixtures containing amino acids have been reported, and many studies have been performed to evaluate their prooxidant effect (Rousseau et al., 1988). Studies on homogeneous systems of linoleic acid with amino acids and tocopherol have shown that the antioxidative effect of tocopherol is considerably stronger in the presence of amino acids and is pH dependent.

The antioxidative effect of amino acids is generally assumed to be of synergistic nature, that is it requires the presence of a primary antioxidant (Rousseau et al., 1988).

Niki et al. (1984) studied the effect of vitamins E and C in inhibiting the oxidation of methyl linoleate in solution: the two vitamins reacted rapidly with the organic free radicals of methyl linoleate. It is widely accepted that the antioxidant properties of these vitamins are partially responsible for their biological activity; in vivo they act synergistically, vitamin E acting as primary antioxidant, and the resulting vitamin E radical then reacting with vitamin C to regenerate vitamin E.

In a systematic study of the synergistic effects of several additives on the antioxidant properties of vitamin E, Rousseau and co-workers induced oxidation of vitamin E, alone or with additives, in heptanol at 80°C, and evaluated the action mechanism in the absence of dissociation of ionizable groups. (Rousseau-Richard et al., 1991) they found that vitamin E alone is oxidized in a step mechanism, whereas vitamin C is oxidized in a short chain mechanism; when vitamins E and C are mixed, vitamin C depresses the oxidation rate of vitamin E, while vitamin E has practically no effect on that of vitamin C. Moreover, some amino acids inhibit the induced oxidation of vitamins E and C, alone or in mixtures, exerting an important synergistic effect on the inhibiting properties of vitamin E—vitamin C mixtures in the radical oxidation of methyl linolenate in the homogeneous phase.

This study, some naturally-occurring amino acids, L-tryptophan, L-alanine, L-cysteine, glycine, a tripeptide, glutathione and *n*-butylamine were added in association with vitamin C to micellar solutions and O/W emulsions of linoleic acid containing vitamin E as primary antioxidant, to evaluate their possible synergistic effect on the inhibition of the lipoperoxidation of linoleic acid.

2. Materials and methods

2.1. Materials

Linoleic acid (LH) was from Aldrich;, α,α'-azoisobutyronitrile (AIBN), isopropylmyristate (IPM), D,L-α-tocopherol (vitamin E), glycine (Gly) were from Fluka; ethylenediaminetetraacetic acid sodium salt (EDTA), L-tryptophan (L-Trp), L-alanine (L-Ala) were from Merck; sodium dodecylsulphate (SDS), L-ascorbic acid (vitamin C), glutathione reduced form (Gsh), L-cysteine (L-Cys), *n*-butylamine (BUT) were from Sigma; Carbomer 940 (Carbopol) was from Biochim; Belsil ADM 6057 (antifoam) was from Wacker Chemie.

2.2. Apparatus

Biological oxygen monitor Model YS 153 equipped with polarographic electrodes (Yellow Spring Instruments Co.); mV/V Module series 2000 mod. 21 (Linear Instruments Corporation); Ultra Turrax Homogenizer T25 (IKA, Janke and Kunkel); UV-visible spectrophotometer mod. DMS 80 (Varian); HPLC apparatus consisting of an UV detector SPD-2A, a pump unit control LC 6A and a C-R3A chromatopac integrator (Shimadzu); 0.71 pH meter with a combination electrode (Beckman).

2.3. Preparation of micellar solutions

Clear micellar systems were obtained mixing fixed amounts of water, SDS and LH. EDTA was added to the water solution as sequestering agent; azo-initiator AIBN was previously dissolved in LH.

The resulting composition was: LH 4.8×10^{-2} M; SDS 5×10^{-1} M; AIBN 3.6×10^{-3} M; EDTA 1×10^{-4} M.

Micellar solutions were also prepared with:

(a) vitamin E: $1.0 - 2.0 - 3.0 - 4.0 - 5.0 - 6.0 \times 10^{-6}$ M

(b) vitamin E: 5.0×10^{-6} M and vitamin C $0.4–0.5–0.625–1.0 \times 10^{-4}$ M

(c) vitamin E: 5.0×10^{-6}M and vitamin C 0.5×10^{-4} M with:

- L-Trp: 8.5×10^{-5} M–1.0×10^{-4} M
- L-Ala: $1.0–1.2 \times 10^{-4}$ M
- L-Cys: 8.5×10^{-4} M–1.0×10^{-4} M
- Gly: $1.0–1.2–1.5 \times 10^{-4}$ M
- Gsh: $7.5–8.5 \times 10^{-5}$ M

In two series of experiments, micellar solutions (volume = 10 ml), to which an antifoaming agent had been added, were brought to pH 5.0 and 7.0 by addition of dilute HCl and NaOH solutions respectively.

2.4. Preparation of O/W emulsions

Emulsions of low viscosity were prepared. AIBN was dissolved in a mixture of IPM and LH at room temperature. The oil phase was then added to a water solution of SDS, Carbopol and EDTA at room temperature under continuous homogenization.

The resulting composition was: LH: 4.8×10^{-2} M; IPM: 0.85 g; SDS: 6.93×10^{-2} M; AIBN: 3.6×10^{-3} M; EDTA: 1×10^{-4} M; Carbopol: 0.024 g.

O/W emulsions were also prepared with:

(a) vitamin E: $3.33–5.0–6.0–8.0–8.67–10.0 \times 10^{-6}$ M

(b) vitamin E: 5.0×10^{-6} M plus vitamin C: $6.67 \times 10^{-6}–1.0–1.33 \times 10^{-5}$ M

(c) vitamin E: 5.0×10^{-6} M plus vitamin C: 1.33×10^{-5} M with:

- L-Trp: $4.33–5.0–5.67 \times 10^{-5}$ M
- L-Ala: $5.0–5.83–6.67 \times 10^{-5}$ M
- L-Cys: $5.0–5.67 \times 10^{-5}$ M
- Gly: $5.0–6.67–8.33–10.0 \times 10^{-5}$ M
- BUT: $4.33–5.0–5.67 \times 10^{-5}$ M
- Gsh: $5.0–5.67–6.67 \times 10^{-5}$ M

O/W emulsions (volume = 15 ml), to which an antifoaming agent had been added, were brought to pH 5.0 or 7.0 by addition of dilute HCl and NaOH solutions.

2.5. Measurements of oxygen uptake

Oxygen uptake was measured for both the series of micellar solutions and emulsions described in the previous sections.

The rate of oxidation of linoleic acid was determined by monitoring the decrease in oxygen concentration in solution, using the biological oxygen monitor and following the method described in previous papers (Carlotti et al., 1991). The reaction vessel, stirred by a magnetic rod-shaped bar, was connected to an oxygen electrode and then thermostated at 45.0 ± 0.1°C to promote decomposition of the azo initiator, which proceeded too slowly at 37.0°C. The measurements were continued for 2 h, to prevent damage to the oxygen probe.

For each system in study the measurement was repeated six times.

2.6. Calculation of dissolved oxygen

The molar concentrations of oxygen solubilized in micellar solutions and in emulsions during the oxidation experiments were determined from the following equation:

$$c_m = a \times p$$

where p is the percentage oxygen content at a specific time and a is a numerical value given by $S/100$, S being the molar solubility of oxygen in water at 45.0°C (2.385×10^{-4} M).

The molar concentration M of consumed oxygen was calculated from the following equation:

$$M = S - c_m.$$

By plotting the molar concentrations of oxygen consumed vs. time, it was possible to obtain

straight-line graphs of oxygen consumption for each system under study, and from these an inhibition period could be calculated for each system, indicating the time lapse before oxygen consumption began.

2.7. Oxidizability of LH in emulsions and in micellar solutions

The rate constant $k_p/(2k_t)^{1/2}$ is generally referred to as the oxidizability of the substrate, where k_p and k_t are propagation and termination rate constants, respectively.

The oxidizability of LH in micellar solutions and in emulsions at pH 5.0 in presence of vitamin E was calculated from the equation:

$$k_p/(2k_t)^{1/2} = R_p/[LH]R_i^{1/2}$$

where: $R_p = -d[O_2]/dt$ and was obtained by plotting the molar oxygen uptake vs. time; R_i is the rate of chain initiation, determined by the conventional inhibitor method (Niki et al., 1984):

$$R_i = n[IH]/t_{inh}$$

where IH is the chain-breaking antioxidant, t_{inh} is the induction period produced by the addition of an inhibitor and n is the stoichiometric number of radicals trapped by each inhibitor. The value of n was taken as 2 (Barclay and Ingold, 1989). R_i was calculated from the slope of the straight line obtained by plotting induction times against the corresponding concentrations of IH (vitamin E).

2.8. Vitamin E consumption

The consumption rates of vitamin E in SDS micellar solutions (a) alone; (b) with vitamin C; (c) with L-Cys; (d) with vitamin C and L-Cys, in the presence of AIBN at 56.0°C at pH 5.0 were determined monitoring vitamin E concentration by HPLC. Chromatographic conditions were: column: Spherisorb C_8 5 $\mu \times 15$ cm; mobile phase: methanol/water 95/5 (v/v); flux: 1 ml/min; UV_{max} 295 nm.

Six micellar solutions with the following concentrations were examined:

Vitamin E: 5.0×10^{-4} M

Vitamin E: 5.0×10^{-4} M plus vitamin C: 5.0×10^{-4}–1.0×10^{-3}–5.0×10^{-3} M

Vitamin E: 5.0×10^{-4} M plus vitamin C: 5.0×10^{-4} M and L-Cys: 2.5×10^{-3}–5.0×10^{-3} M

Each micellar solution had identical fixed composition:

SDS: 5.0×10^{-1} M; AIBN: 4.68×10^{-3} M; EDTA: 1×10^{-4} M.

2.9. Determination of pK_a of L-Trp in SDS micelles by potentiometric titration

Triplicate determinations were performed in a thermostated cell at 25.0 ± 0.1°C using a pH meter with a combination electrode, standardized with Ingold buffers at pH 4.00 and 7.00. The time taken for each pH reading was regulated by the microprocessor in the Beckman meter which displayed the steady-state value when the rate of change with time reached a minimum value.

The general procedure was to prepare a solution with 1.0×10^{-2} M L-Trp and 1.0×10^{-1} M NaCl; 25.00 ml of this solution were titrated with 5.0×10^{-1} M HCl under a nitrogen atmosphere.

The measurements were replicated in the same conditions, in the presence of 1.0×10^{-1} M SDS; L-Trp and SDS were stored over a desiccant and all the solutions were prepared with CO_2-free water.

3. Results and discussion

Tocopherols are well known to act as antioxidants both in vitro and in vivo (Cillard and Cillard, 1980) by scavenging chain propagating peroxy-radicals. In vitro, their antioxidant efficacy decreases in the order $\delta > \gamma > \alpha$, whereas α-tocopherol is most effective as vitamin E (Ikeda and Fukuzumi, 1977). The antioxidant effect of primary antioxidants, such as the tocopherols, can be enhanced by the addition of various products, usually classified as synergists: these include amino acids, whose mechanism of action is not yet fully understood.

In a previous paper (Carlotti et al., 1991), the autoxidation of linoleic acid was studied in microemulsions and in emulsions stabilized by non

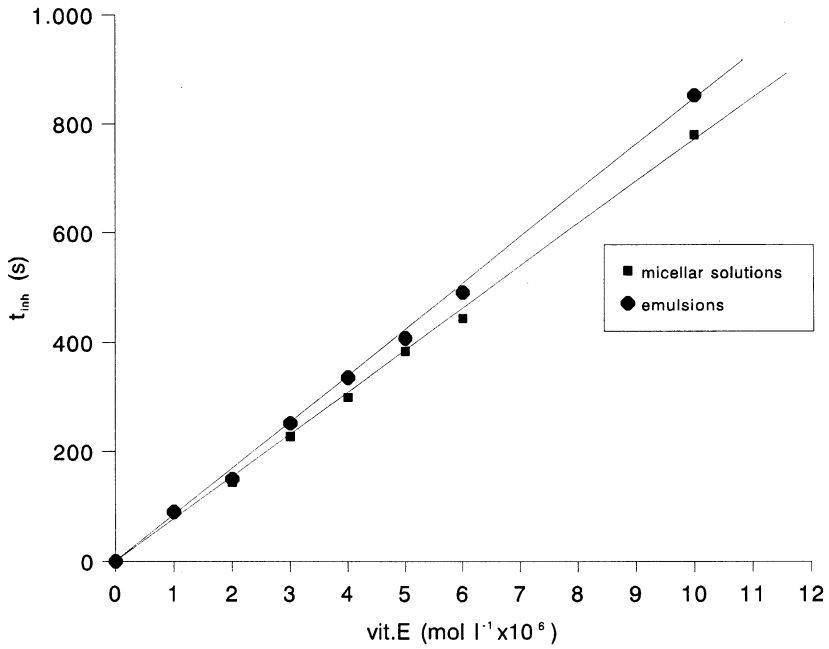

Fig. 1. Induction times of auto-oxidation of LH in micellar solutions and in O/W emulsions at pH 5.0 as a function of vitamin E concentration.

ionic surfactants, and the pro- or antioxidant behaviour of α-tocopherol was evaluated.

In the actual research, we noted that vitamin E was able to inhibit LH oxidation in micellar solutions and in O/W emulsions.

The results of oxidizability measurement of LH in both systems justified the comparison of antioxidant and synergistic behaviours of several substances in two different systems such as micellar solutions and O/W emulsions. The oxidizability of LH, calculated by the inhibitor method (Niki et al., 1984) described above, was similar in micellar solution and in O/W emulsion (1.89×10^{-3} and 1.29×10^{-3} $M^{-1/2}/s^{1/2}$ respectively) (Fig. 1). The lower value obtained in O/W emulsions could probably be ascribed to a slowed diffusion of oxygen into O/W systems, probably because these are more viscous than micellar solutions.

In micellar solution at pH 5.0 the oxidation of LH initiated by AIBN proceeded at a constant rate of oxygen uptake with a 60 s induction period (Table 1). The addition of vitamin C produced a very poor inhibiting action ($t_{inh} = 100$ s). On the other hand, vitamin E showed a remarkable antioxidant activity in micellar systems as a function of its concentration, slowing the oxygen consumption rate and increasing the induction period up to six times at 5.0×10^{-6} M. A certain synergistic effect was exerted by vitamin C at the

Table 1
Induction times (t_{inh}) of oxygen consumption by oxidation of LH 4.8×10^{-2} M in SDS micellar solutions at pH 5.0 (45°C) in the presence of vitamin C or increasing concentrations of vitamin E

Tested substance (M)	t_{inh} (s)*
None	60 (8)
Vitamin C 5.0×10^{-5}	100 (11)
Vitamin E 1.0×10^{-6}	90 (9)
Vitamin E 2.0×10^{-6}	170 (13)
Vitamin E 3.0×10^{-6}	240 (18)
Vitamin E 4.0×10^{-6}	300 (19)
Vitamin E 5.0×10^{-6}	360 (21)
Vitamin E 6.0×10^{-6}	380 (22)

* Values in brackets are S.D. ($n = 6$).

Table 2
Induction times (t_{inh}) of oxygen consumption by oxidation of LH 4.8×10^{-2} M in SDS micellar solutions at pH 5.0 (45°C) in the presence of vitamin E 5.0×10^{-6} M and increasing concentrations of vitamin C (initiator = AIBN)

Tested substance (M)	t_{inh} (s)*
None	360 (21)
Vitamin C 4.0×10^{-5}	380 (32)
Vitamin C 5.0×10^{-5}	430 (29)
Vitamin C 6.25×10^{-5}	480 (26)
Vitamin C 1.0×10^{-4}	540 (30)

* Values in brackets are S.D. ($n = 6$).

higher increasing concentrations in micellar solutions containing vitamin E 5.0×10^{-6} M (Table 2), probably because the vitamin C, present in the aqueous phase, served to regenerate vitamin E, located in the micellar phase (Barclay et al., 1983). The co-presence of vitamin C (hydrophilic) and vitamin E (hydrophobic) extended the latter's effective inhibition period. If the polar phenolic head group Ar-OH of vitamin E were positioned in the micelles near the aqueous phase, the derived radical ArO· could readily regenerate vitamin E by the transfer of a hydrogen atom from ascorbate positioned in the aqueous phase.

The addition of amino acids and Gsh to a micellar solution containing 5.0×10^{-6} M vitamin E plus 5.0×10^{-5} M vitamin C prolonged induction times in all cases (Table 3). The synergistic action was particularly remarkable for L-Cys, L-Trp and Gsh, which markedly prolonged the induction periods, at concentrations lower than were needed for L-Ala and Gly. (550 s for 1.0×10^{-4} M L-Cys; 540 s for 1.0×10^{-4} M L-Trp; 540 s for 8.5×10^{-5} M Gsh; 500 s for 1.2×10^{-4} M L-Ala; 470 s for 1.2×10^{-4} M Gly). The oxygen uptake rates did not differ significantly from that obtained in the micellar solution with vitamin E and vitamin C. It is noteworthy that the presence of amino acids alone was not sufficient to prolong the inhibition period of oxidation of LH in the micellar systems studied.

The inhibition effect of vitamin E on the oxidation of LH in micellar solutions was also seen at pH 7.0. The addition of 5.0×10^{-6} M vitamin E

Table 3
Induction times (t_{inh}) of oxygen consumption by oxidation of LH 4.8×10^{-2} M in SDS micellar solutions at pH 5.0 (45°C) in the presence of vitamin E 5.0×10^{-6} M, vitamin C 5.0×10^{-5} M and amino acids or Gsh (initiator = AIBN)

Tested substance	Concentration (M)	t_{inh} (s)*
Gsh	7.5×10^{-5}	460 (27)
Gsh	8.5×10^{-5}	540 (33)
L-Trp	8.5×10^{-5}	470 (28)
L-Trp	1.0×10^{-4}	540 (31)
L-Ala	1.0×10^{-4}	470 (26)
L-Ala	1.2×10^{-4}	500 (32)
L-Cys	8.5×10^{-5}	490 (26)
L-Cys	1.0×10^{-4}	550 (38)
Gly	1.0×10^{-4}	390 (27)
Gly	1.2×10^{-4}	470 (31)
Gly	1.5×10^{-4}	590 (36)

t_{inh} of LH alone = 60 s (8); in the presence of vitamin C 5.0×10^{-5} M = 100 s (11); in the presence of vitamin E 5.0×10^{-6} M = 360 s (21) in the presence of vitamin E 5.0×10^{-6} M + vitamin C 5.0×10^{-5} M = 430 s (29).
* Values in brackets are S.D. ($n = 6$).

to a micellar solution at pH 7.0 prolonged the induction period 4.5 times (from 80 to 360 s) and slowed the oxygen consumption rate, while 5.0×10^{-5} M vitamin C was not only ineffective, but also suppressed the antioxidant activity of vitamin E ($t_{inh} = 110$ s) (Table 4). Surprisingly, the introduction of Gsh, L-Trp, L-Ala, L-Cys, Gly or BUT did not modify the trend of oxygen consumption further (Table 4).

Table 4
Induction times (t_{inh}) of oxygen consumption by oxidation of LH 4.8×10^{-2} M in SDS micellar solutions at pH 7.0 (45°C) in the presence of vitamin E 5.0×10^{-6} M, vitamin C 5.0×10^{-5} M and amino acids or Gsh (initiator = AIBN)

Tested substance	Concentration (M)	t_{inh} (s)*
Gsh	8.5×10^{-5}	110 (10)
L-Trp	1.0×10^{-4}	100 (9)
L-Ala	1.0×10^{-4}	80 (8)
L-Cys	1.0×10^{-4}	100 (8)
Gly	1.5×10^{-4}	130 (11)

t_{inh} of LH alone = 80 s (9); in the presence of vitamin C 5.0×10^{-5} M = 80 s (9); in the presence of vitamin E 5.0×10^{-6} M = 360 s (24); in the presence of vitamin E 5.0×10^{-6} M + vitamin C 5.0×10^{-5} M = 110 s (12).
* Values in brackets are S.D. ($n = 6$).

Table 5
Induction times (t_{inh}) of oxygen consumption by oxidation of LH 4.8×10^{-2} M in O/W emulsions at pH 5.0 (45°C) in the presence of vitamin E 5.0×10^{-6} M, vitamin C 1.33×10^{-5} M, amino acids, BUT and Gsh (initiator = AIBN)

Tested substance	Concentration (M)	t_{inh} (s)*
Gsh	5.67×10^{-5}	620 (38)
Gsh	6.67×10^{-5}	660 (45)
L-Trp	4.33×10^{-5}	790 (49)
L-Trp	5.00×10^{-5}	860 (47)
L-Ala	5.83×10^{-5}	620 (39)
L-Ala	6.67×10^{-5}	700 (43)
L-Cys	5.00×10^{-5}	710 (44)
L-Cys	5.67×10^{-5}	730 (49)
Gly	6.67×10^{-5}	610 (44)
Gly	8.33×10^{-5}	640 (38)
L-Glu	5.00×10^{-5}	540 (37)
L-Glu	5.67×10^{-5}	600 (42)
BUT	4.33×10^{-5}	400 (30)
BUT	5.67×10^{-5}	610 (39)

t_{inh} of LH alone = 130 s (10); in the presence of vitamin C 1.33×10^{-5} M = 250 s (18); in the presence of vitamin E 5.0×10^{-6} M = 400 s (24); in the presence of vitamin E 5.0×10^{-6} M + vitamin C 1.33×10^{-5} M = 590 s (36).
* Values in brackets are S.D. ($n = 6$).

In O/W emulsion at pH 5.0 (Table 5), the oxidation of LH initiated after an induction period (130 s) more than twice that in micellar solution (60 s), probably because the viscosity of the emulsion, higher than that of micellar solutions, slowed down access to oxygen in the lipophilic dispersed phase. The antioxidant behaviour of vitamin E was similar to that in micellar solutions at the same pH: 5.0×10^{-6} M vitamin E produced a t_{inh} of 400 s; moreover, induction time increased further, to a value (780 s) 6-fold the initial one at an 1.0×10^{-5} M vitamin E concentration.

Other than in micellar solutions at the same pH value, the presence of vitamin C alone (1.33×10^{-5} M) exerted an inhibiting action (250 s) on the autoxidation of LH. The addition of both vitamin E (5.0×10^{-6} M) and vitamin C (1.33×10^{-5} M) further prolonged the induction time (590 s).

Among the amino acids examined, only L-Cys and L-Trp exerted a noteworthy synergistic effect on the inhibiting action of vitamin E–vitamin C (Table 5), and the induction time was prolonged in function of their concentration (1.46 and 1.23 times, for 5.0×10^{-5} M L-Trp and 5.67×10^{-5} M L-Cys, respectively). Values of 6.67×10^{-5} M Gsh and 6.67×10^{-5} M L-Ala also had some synergistic effect (1.12 and 1.19 times, respectively); on the other hand, Gly and BUT were only slightly effective as synergists at high concentration values (Table 5). B//ut, carrying only the amino group, was used for comparison with amino acids in investigating their action mechanism.

At pH 5.0, the over-all inhibition activities of the systems studied were more pronounced in O/W emulsions than in micellar solutions.

In O/W emulsions at pH 7.0, the inhibition effect of 5.0×10^{-6} M vitamin E was similar to that at pH 5.0, with induction time somewhat prolonged (from 180 to 380 s), though less so than at pH 5.0. Vitamin C alone did not significatively modify the induction period of the oxidation of LH (Table 6), and its synergistic effect in the presence of vitamin E was very poor (from 380 to 460 s). Moreover, vitamin C suppressed the synergistic effect of amino acids, there being no increase in induction times when the amino acids were added to emulsive systems containing vitamin C–vitamin E (Table 6). Indeed, as previously seen for micellar solutions, at pH 7.0 neither the amino acids studied nor BUT nor Gsh were able to further modify the antioxidant activity of the

Table 6
Induction times (t_{inh}) of oxygen consumption by oxidation of LH 4.8×10^{-2} M in O/W emulsions at pH 7.0 (45°C) in the presence of vitamin E 5.0×10^{-6} M, vitamin C 1.33×10^{-5} M, amino acids, BUT and Gsh (initiator = AIBN)

Tested substance	Concentration (M)	t_{inh} (s)*
None	—	460 (29)
Gsh	5.67×10^{-5}	430 (31)
L-Trp	5.67×10^{-5}	460 (30)
L-Ala	6.67×10^{-5}	370 (29)
L-Cys	5.67×10^{-5}	440 (24)
Gly	1.00×10^{-4}	420 (25)
BUT	5.00×10^{-5}	400 (25)

t_{inh} of LH alone = 180 s (13); of LH + vitamin E 5.0×10^{-6} M = 380 s (21); of LH + vitamin C 1.33×10^{-5} M = 170 s (11).
* Values in brackets are S.D.

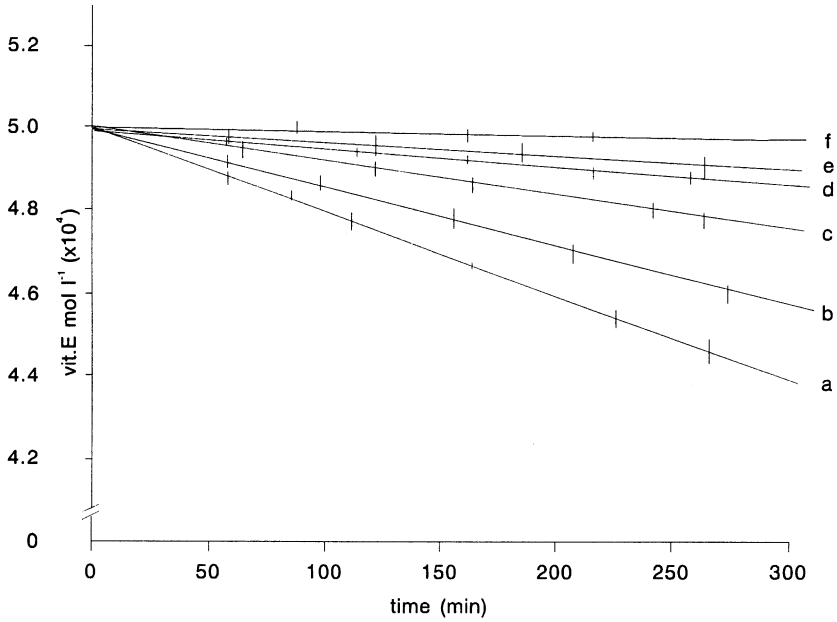

Fig. 2. Consumption of vitamin E 5.0×10^{-4} M in SDS micellar solution in the presence of AIBN at 56°C at pH 5.0 alone and with vitamin C and L-Cys. a, Vitamin E alone; b, vitamin E + vitamin C 5.0×10^{-4} M; c, vitamin E + vitamin C 1.0×10^{-3} M; d, vitamin E + vitamin C 2.5×10^{-3} M; e, vitamin C 5.0×10^{-3} M; f, vitamin E + vitamin C 5.0×10^{-4} M + L-Cys 5.0×10^{-3} M.

system vitamin E–vitamin C. Nor did the amino acids, Gsh, BUT by themselves act as antioxidant agents either at pH 5.0 or pH 7.0.

Vitamin E oxidation to tocopheryl radicals in micellar solution at pH 5.0 was studied, to explain the inhibition mechanisms of several antioxidants, at 56.0°C to increase the rate of formation of tocopheryl radical from vitamin E. The results obtained confirmed the synergistic action of vitamin C and L-Cys (Fig. 2); with either additive, the disappearance of vitamin E slowed increasingly as the concentration of additive increased. Vitamin E alone, and vitamin E plus 5.0×10^{-4} M vitamin C decreased by 16 and 13%, respectively, in 6 h in the experimental conditions. In the presence of 5×10^{-4} M vitamin C plus 5×10^{-3} M L-Cys, vitamin E was completely restored. L-Cys alone, at a concentration 5×10^{-3} M, was able to restore vitamin E (Fig. 3).

Among the additives studied, L-Trp was the most effective synergist, both in micellar solutions and in emulsions at pH 5.0. This could be explained by assuming a distribution of L-Trp, in the micellar core or in the O/W interface, higher than that of L-Cys, L-Ala, Gly, in line with the data of apparent partition coefficients *n*-octanol/water (D) determined at various pHs (El Tayar et al., 1992). In detail, log *D* values were -3.01 for Gly, -2.77 for L-Ala, -2.57 for L-Cys and -1.15 for L-Trp, calculated from the mean values obtained at pH 5.0, 6.0, 7.0. The increase in lipophilicity might favour the location of L-Trp at the O/W interface in emulsions, or at the micellar core/bulk solution interface in micellar solutions, where the oxidizable substrate (LH) might be present. L-Cys was more effective than L-Ala as synergist in spite of quite similar log D values; this apparent discrepancy could be explained by assuming that the thiolic group, whose antioxidant activity is well known, could exert an inhibition effect on LH oxidation. Also in the case of Gsh, the inhibition effect could be ascribed to the presence of the SH group.

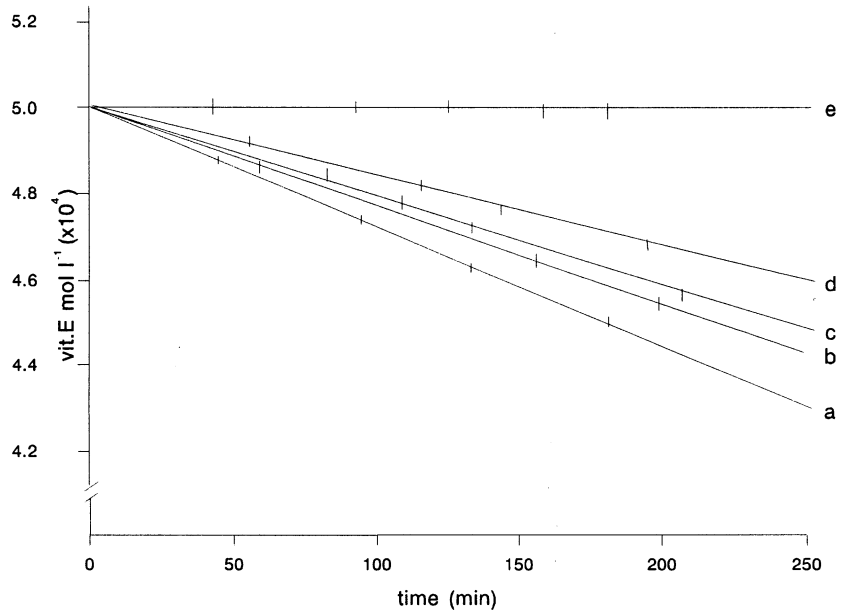

Fig. 3. Consumption of vitamin E 5.0×10^{-4} M in SDS micellar solution in the presence of AIBN at 56°C at pH 5.0 alone and with increasing concentrations of L-Cys. a, Vitamin E alone; b, vitamin E + L-Cys 2.5×10^{-3} M; c, vitamin E + L-Cys 5.0×10^{-3} M; d, vitamin E + L-Cys 1.0×10^{-2} M; e, vitamin E + L-Cys 3.0×10^{-2} M.

The lack of synergistic effect of the amino acids that was found at pH 7.0 could not be explained from data in the literature, showing that, at the pHs considered (5.0, 7.0), the apparent lipophilicity of L-Cys, L-Trp, L-Ala, Gly does not vary appreciably, the isoelectric pHs of these amino acids all being in the range 5.97–6.25.

According to some workers (Kahledi and Rodgers, 1990), the pK_a values of carboxylic groups of amino acids vary as a function of the dissolution medium. Shifts in pK_a values occur in several amino acids when solubilized in 0.1 M SDS; shifts were more significant for amino acids with hydrophobic or basic groups, which can be submitted to electrostatic attraction by the negatively charged SDS micelles, while more hydrophilic amino acids showed less pronounced shifts. pK_a of L-Trp determined in SDS micelles was 4.59 (Kahledi and Rodgers, 1990), being 2.32 in 0.1 M NaCl (shift = + 2.27). On the other hand, dissociation of the amino group was not influenced by micelles.

According to data in the literature (Kahledi and Rodgers, 1990), in SDS micellar solution at pH 5.0 (the experimental conditions of this study) L-Trp should be 72% in the dissociated form, and at pH 7.0 100% in the dissociated form, with regard to the carboxylic group. It follows that, the association of L-Trp to SDS anionic micelles could be favoured at pH 5.0 rather than at pH 7.0, where there might be repulsive interactions between dissociated L-Trp and SDS micelles.

To confirm the hypothesis formulated from data in the literature pK_a of L-Trp was determined in this study in SDS micellar solutions by the potentiometric technique described in the methods section: pK_a was found to be 4.21, in good agreement with the literature. Moreover, some researchers (Caselli and Mangone, 1992), who have studied the partition of L-Trp, L-Ala and L-Cys between water saline and an organic reverse micellar solution have noted a maximum extractability by the organic phase for L-Trp, more pronounced at pH 5.0 than at pH 7.0. Both acid dissociation and partition patterns of L-Trp in

micellar solutions seem to favour the location of the amino acid at the O/W or micellar core/bulk solution interphase, where the synergistic action toward antioxidant activity of vitamin E can more easily be exerted (Marcuse, 1962).

Several researchers (Rousseau et al., 1988) have undertaken studies on specific model systems aimed at determining the anti- or pro-oxidative behaviour of some individual amino acids added to linoleic acid. The discrepancies in results concerning the effect of amino acids on lipid oxidation achieved in different experimental systems stem from the difference in temperature, lipid concentration, type of emulsifier, pH etc. The mechanism of the antioxidative or prooxidative effect of amino acids is still controversial. Some workers have noted (Riisom et al., 1980) that the prooxidative activity of amino acids can be attributed to the presence of the α-amino group in the protonated form (NH_3^+, which accelerates the autooxidation, the NH_2 group having the reverse effect. On the other hand, amino acids can act as antioxidant via a number of mechanisms, such as complexation of peroxyl radicals and of cupric and ferric ions; the latter seems not to be predominant in the systems studied here as EDTA might inactivate lipoperoxidation reactions initiated by heavy metals. The mechanism that the studies by Marcuse (1962) support involves the recovery of vitamin E from its radical by amino acids, which act as donors of H· radical from the NH_2 group. At pH 5.0, although the NH_2 group of the amino acids is in the protonated form in an aqueous medium, less hydrophilic amino acids, such as tryptophan, are partially extracted at the interface, where vitamin E also is located: the interfacial microenviroment might be considered similar to a non-aqueous medium, in which NH_2 groups could be present in the non-dissociated form and can therefore recover vitamin E from its radicals.

The antioxidant effect of vitamin C, less marked at pH 7.0 than at pH 5.0, is apparently opposite to what could be expected from Nernst equation: Eq. (1)

$$E = E^0 - 0.05916/2 \log[H_2A]/[D][H^+]^2 \quad (1)$$

where D is the dehydroascorbic acid (oxidized form)

H_2A = ascorbic acid (reduced form)

in the reaction: $D + 2H^+ + 2e^- \leftrightarrow H_2A$

Moreover, the trend of the reduction potential of vitamin C (Ruiz et al., 1977) shows a decrease at increasing pH values, indicating that the antioxidant power of vitamin C increases with pH.

The opposite behaviour, noted both in SDS solubilized and emulsified systems, could be ascribed to a repulsive force between the dissociated form of vitamin C ($pK_a = 4.17$) especially present at pH 7.0, and the negative charge of SDS micelles, opposing the regeneration of vitamin E by vitamin C in the micellar phase.

The unsuccessful results obtained at pH 7.0 could be furthermore explained from data in the literature (Allan and Wood, 1970): the easier oxidation of ascorbic acid to dehydroascorbic acid, and the reaction of the ascorbic acid radical with oxygen and consequent formation of free HO· radicals, could encourage a prooxidant activity of ascorbic acid, and might also deactivate the inhibiting effect of amino acids on lipoperoxidation.

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